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Research Article

Advances in conservation physiology and *ex situ* propagation strategies for the rare moss *Podperaea krylovii* (Amblystegiaceae, Bryophyta)

Bojana Z. Jadranin[1], Marija V. Ćosić[1], Djordje P. Božović[1], Milorad M. Vujičić[1,2], Michael S. Ignatov[3,4], Aleksey V. Troitsky[5], Aneta D. Sabovljević[1,2], Marko S. Sabovljević[1,2,6]

1 *Institute of Botany and Botanical garden "Jevremovac", Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia*
2 *Center of Plant Biotechnology and Conservation (CPBC), Takovska 43, 11000 Belgrade, Serbia*
3 *Tsitsin Main Botanical Garden, Russian Academy of Sciences, Botanicheskaya Str. 4, Moscow 127276, Russia*
4 *Faculty of Biology, Lomonosov Moscow State University, Leninskie Gory Str. 1–12, Moscow 119234, Russia*
5 *Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia*
6 *Department of Plant Biology, Institute of Biology and Ecology, Faculty of Science, Pavol Jozef Šafárik University in Košice, Mánesova 23, 040 01 Košice, Slovakia*
Corresponding author: Marko S. Sabovljević (marko@bio.bg.ac.rs)

Abstract

This study investigates the growth and micropropagation of the rare moss *Podperaea krylovii*, under *in vitro* conditions. The effects of different growth media, plant growth regulators, and sugars were tested to optimize conditions for efficient propagation and to gain a better understanding of the species' biology and the cultivation requirements of this species. The results indicate that the KNOP medium is most effective for the propagation of *P. krylovii*, with prostrate-oriented explants having a higher index of multiplication compared to upright-oriented explants. The addition of auxin had no significant effect on species multiplication and development in comparison to auxin-free media. Conversely, cytokinin inhibited shoot formation as well as protonemal development, suggesting that its use individually was not suitable for the species' development under axenic conditions. The sugars tested showed no positive effect on new shoot formation, suggesting that no exogenous carbon source is required and that this species keeps autotrophy in axenic cultures. These results provide a basis for future research on the propagation and cultivation of *P. krylovii* and emphasize the need for specific cultivation protocols. The *in vitro* multiplicated moss was successfully acclimatised and propagated *ex vitro* in a xenic environment. This enables further successful *ex situ* conservation of the species as well as commercial use as aquatic plants, for example in aquaria.

Key words: Acclimation, axenic culture, bryophytes, micropropagation, plant growth regulators, sugars

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Introduction

The amblystegian pleurocarp moss *Podperaea krylovii* (Podp.) Z. Iwats. & Glime (synonym *Chrysohypnum krylovii* Podp.; syn. *Campylium krylovii* (Podp.) Laz.) was initially described as a genus and species new to science, belonging to the family Hypnaceae (Iwatsuki and Glime 1984). However, later molecular studies showed

that this species is more closely related to members of the family Amblystegiaceae (Ignatov and Milyutina 2011). The species is rare, but morphologically and molecularly confirmed as a good taxon. Although it is also easily recognized in the field by its superficial resemblance to *Campylium sommerfeltii* Myr., but easily distinguished by the double-celled teeth on the leaf margin, not so many ecological and biological features are known for this species due to its rarity and the rather small number of records. The currently known distribution area includes south Siberia (westward to Altai), the Russian Far East, northern Japan (Hokkaido), and northeastern China (Ignatov and Afonina 1992; Ignatov et al. 1996; Ignatov and Milyutina 2011). The records from the Inner Mongolia autonomous District in China led to the description of the second species of this genus, namely *P. baii* Ignatov (both morphologically and molecularly confirmed) (Ignatov and Milyutina 2011).

The biology of the species is rather poorly known and due to its rarity, very little can be found in the literature about this species. For conservation purposes and fundamental issues, such species are of high priority both for biological feature studies (e.g. Rincon 1993; He et al. 2016; Pakeman et al. 2019; Calleja et al. 2022) and biotechnology investigations (e.g. González et al. 2006; Mallón et al. 2007; Decker and Reski 2020). Even in the original description (Iwatsuki and Glime 1984) the ecological characteristics are not given. It inhabits a variety of habitats, including eroded soils on slopes in open places, areas near roads, sites under loose canopy in deciduous forests, soils at the base of cliffs, rock outcrops in non-dense forests, soils above fallen logs in valleys, and also slopes by ponds (Iwatsuki and Glime 1984; Ignatov et al. 2022). The same author gave the altitude of occurrence as 70–450 m, rarely up to 900 m. The species is known to be autoicous (Iwatsuki and Glime 1984) and to produce sporophytes relatively frequently. It seems that the species does not remain in the same location for long, as repeated visits to the same site often do not confirm the presence of the species (pers. comm. Ignatov). The species is regarded as data deficient (DD) under the IUCN criteria. However, its survival may be considered under threat due to habitat loss and sensitivity to environmental and climatic changes.

This study aims to document the biology and developmental features of the species, bearing in mind that this species is rarely empirically documented in remote and uninhabited areas of North Asia. These can be successfully used in the action plans for *ex situ* conservation of the rare moss *P. krylovii* by optimizing axenic and xenic *ex situ* propagation if needed. Indeed, bryophyte conservation can be assessed as neglected in comparison to tracheophytes, and bearing in mind that this group is very heterogeneous and less known, it is not easy to apply biotechnics and biotechnology known to be used in vascular plants.

In this work, we addressed the following key questions: (1) What is the optimal explant orientation and growth medium type for micropropagation of the species under axenic conditions? (2) What are the effects of the exogenous addition of selected plant growth regulators (PGRs) to the growth medium on the morphogenesis of the species and can explant orientation modify their effects? (3) How do exogenously added different sugars (carbon sources and signaling compounds) modify morphogenesis and can they enhance the propagation of the species? (4) Can leafy gametophores be used for successful acclimation and xenic propagation *ex situ* and/or commercially as aquarium plants, e.g. as a conservation method?

Methods

Plant material and *in vitro* culture establishment

The plant material used in this study was obtained from specimens grown under terrarium conditions at Lomonosov University in Moscow by A. Troitsky (August 02, 2013), and previously collected in Primorsky Territory, Russia. This specimen is deposited in the MHA (Herbarium of the Main Botanical Garden of the Russian Academy of Sciences), Russia, as voucher Ignatov #07-220 and was also used for sequencing [Gen-bank #JN896329]. This accession was used for *in vitro* (axenic) experimentations, while moss material from, Lozovyi Range, Partizansk Distr. (Russia), voucher MHA901589, September 08, 2013, Ignatov M.S., Ignatova E.A., Malashkina E.V., #13-1856 (S-facing slope, open oak stand with *Lespedeza bicolor* Turcz., 43°00'N, 133°00'E) was used for xenic propagation of plant material.

Surface sterilization of both gametophytes and sporophytes was performed with different concentrations of ethanol (50% and 90%), sodium hypochlorite (NaOCl) (1%, 3%, 5%, and 7%), and sodium dichloroisocyanurate (NaDCC) (1%, 3%, 5%, and 7%) for different time periods (1, 3, 5, and 7 minutes). While the other solutions tested were lethal to both the gametophytes and spores or were ineffective in eliminating contamination by xenic organisms and contaminants, the 3% NaDCC solution used for 5 minutes proved to be the most effective solution for sterilizing the plant material.

Once the axenic culture of *P. krylovii* was established, individual 1 cm long and decapitated explants were used for the experiments, which included different media types with or without supplementation (e.g. plant growth regulators or sugars). Decapitation (removing the tip of the plantlets) eliminates dividing cells and minimizes possible errors in the measurement of regeneration and morphological parameters. This ensures that each explant has an equal chance to de-differentiate and initiate the formation of a new meristem and tips. The explants were placed in different treatments on the media whose pH was adjusted to 5.8 before being autoclaved at 121 °C for 30 minutes. All treatments used in this study consisted of four individual explants in five separate Petri dishes, i.e. a total of 20 gametophores per treatment. The explants were cultured under axenic conditions in sterile Petri dishes at a temperature of 18 ± 2 °C and a humidity of 60–70% and exposed to a long-day light cycle (16 h light/8 h dark) with fluorescent tubes (Tesla Pančevo) providing a light intensity of 50 μmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD).

Experimental design

This study was conducted in three different experimental setups (see Fig. 1). The “Medium type experiment” focused on examining the effects of different growth media on the growth and development of the moss *P. krylovii*. Explants were placed on solid KNOP medium (Knop 1865), solid half-strength Murashige & Skoog medium (MS/2) (Murashige and Skoog 1962; Ćosić et al. 2025a), and solid BCD medium (Ćosić et al. 2025a). The plantlets were positioned both upright and prostrate on the media to investigate whether the position of the explants influences morphogenesis.

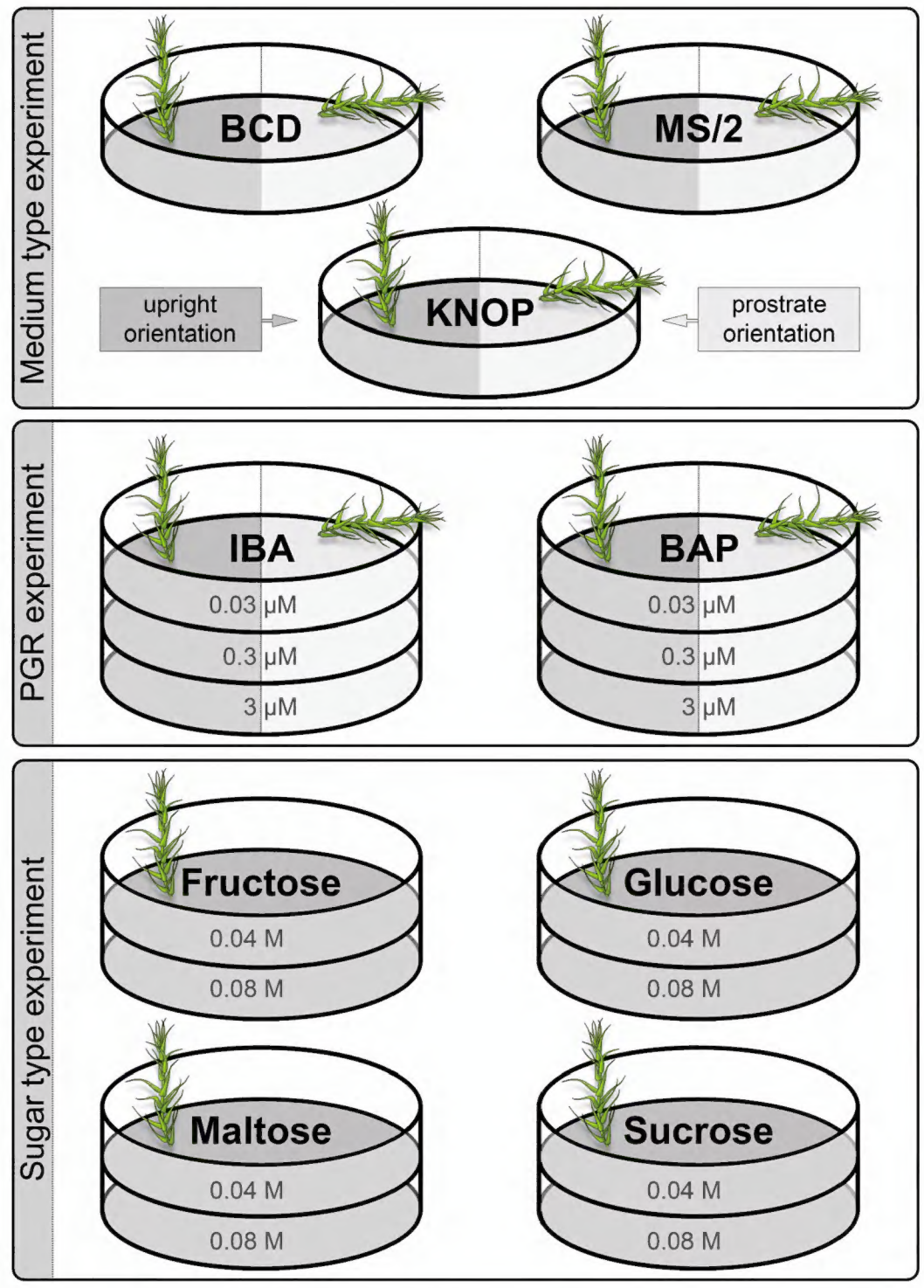

Figure 1. A diagram of the experimental design. The "Medium type experiment" included three different solid media types (KNOP, MS/2 and BCD) on which the explants were placed in two positions, either upright or prostrate. The "PGR experiment" included experimental setups with different concentrations (0.03, 0.3, and 3 µM) of IBA and BAP added to the KNOP medium on which the explants were placed in two positions, either upright or prostrate. In the "Sugar type experiment", different concentrations (0.04 and 0.08 M) of four sugars (fructose, glucose, maltose and sucrose) were added to the KNOP medium on which the explants were placed exclusively in upright position. In all experiments performed, each treatment consisted of four explants in five Petri dishes (a total of 20 explants per treatment).

The "PGR experiment" investigated the effect of plant growth regulators (PGRs), in particular auxins and cytokinins, on the growth and development of the target moss. Different concentrations (0.03, 0.3, and 3 µM) of IBA (indole-3-butyric acid) and BAP (6-benzylaminopurine) were applied exogenously to the KNOP medium on which the explants were placed in both upright and prostrate positions. The KNOP medium was chosen because the gametophores developed well and formed numerous new shoots without protonema as inferred from the results of the "Medium type experiment".

In the "Sugar type experiment", different concentrations (0.04 and 0.08 M) of four sugars were added individually to the KNOP minimal medium with aim to document its action as signal molecules and/or source of carbon in further development. The primary sugar concentration used in this experiment was

set to half-strength (15 g/L) compared to the concentration commonly used in MS medium (30 g/L) for all four sugars, as bryophytes do not require high concentrations of carbon sources for their growth *in vitro*. Since the half-strength molar concentration is 0.04 M for maltose and sucrose and 0.08 M for fructose and glucose, we used both concentrations for all tested sugars to enable a comparative analysis of the results.

After 4 weeks, the morphogenetic changes were observed, including the formation of new shoots on the initial explant (i.e. index of multiplication), the diameter of the secondary protonema and the survival rate. The morphological parameters were measured and photographed using a Leica MZ stereomicroscope (Leica MZ 7.5, Bi-Optic Inc., Santa Clara, CA, USA).

Acclimation of *in vitro* propagated *Podperaea krylovii*

The optimization of *in vitro* propagation enabled sufficient biomass production and acclimation of the investigated species. The plantlets were transferred from axenic *in vitro* to controlled xenic laboratory conditions and acclimation lasted for four weeks. The moss material was placed in Magenta containers filled with distilled water on white terrazzo marble gravel chippings (5–8 mm in size). The environmental conditions were at a temperature of 18 ± 2 °C, relative humidity of 100%, and continuous light with a 24-hour photoperiod. The light source consisted of fluorescent tubes (Phillips Lighting, Amsterdam, The Netherlands) with a flux density of 35 μmol m^{-2} s^{-1}.

Additionally, translocation test to controlled conditions in a growth chamber (Sanyo Environmental Test Chamber MLR-352H) on the natural substrate to xenically propagate the material.

Statistical analysis

The entire statistical analysis was conducted using the R programming language (v. 4.3.2) (R Core Team 2023). The first step of the analysis consisted of preliminary data exploration using the Shapiro-Wilk normality test and Levene test for homogeneity of variance, revealing that normality and homoscedasticity were violated across the groups. Consequently, for all of the measured parameters in all of the experiments, a nonparametric factorial ANOVA was performed employing the Align Rank Transform (ART) procedure (Wobbrock et al. 2011; Elkin et al. 2021) from "ARTool" R package (Kay et al. 2021). The factorial models were created using the "art" function and the significance of the main effects and interactions was evaluated with the "anova" function, after which the contrast test was performed using the "art.con" function of the mentioned R package.

Results

The effects of explant position and growth medium type on the morphogenesis of *Podperaea krylovii*

In the initial experiment ("Medium type experiment"), the main effects of explant orientation (EO) and medium type (MT) significantly affected both of the morphogenesis parameters i.e. the index of multiplication (IM) and

the diameter of secondary protonema patch (DP) ($P < 0.01$ and $P < 0.001$, respectively) in tested species *P. krylovii* (Table 1). Furthermore, a significant interaction of the two factors (EO × MT) was observed for the DP ($P < 0.001$), but not for the IM (Table 1), suggesting that the explant orientation modifies the effect of the different medium types on the development of the secondary protonema.

The highest IM was observed in plants grown on KNOP medium in a prostrate explant orientation, followed by plants on the same medium in an upright orientation, while the lowest IM values were documented in plants grown on MS/2 medium (Fig. 2A). Moreover, a clear distinction among the three tested media types was evident, with all showing significantly different IM values in both prostrate and upright explant orientations ($P < 0.05$). However, no significant differences were observed between the two orientations within any of the media types (Fig. 2A). Interestingly, for all tested media types, plants grown in an upright orientation exhibited lower IM values compared to their prostrate counterparts although these differences were not statistically significant (Fig. 2A).

Concerning the secondary protonema development, the highest DP values were observed in plants grown on BCD medium in an upright orientation, with all other DP values for any combination of explant orientation and medium type being significantly lower ($P < 0.05$) (Fig. 2B). Interestingly, plants grown on KNOP medium in a prostrate explant orientation did not develop any secondary protonema (Fig. 2B). This unique response, together with the highest IM values for this combination of explant orientation and medium type (prostrate orientation of explants grown on KNOP medium) suggest this combination is optimal for biomass production of *P. krylovii*.

Explants grown on KNOP and BCD media in both prostrate and upright orientation formed a remarkable number of gametophores (Fig. 3A, B, D, E) compared to those grown on MS/2 medium (Fig. 3C, F). On the other hand, secondary protonema developed mainly in plants positioned upright on the media (Fig. 3D–F). However, when placed prostrate, plants grown on BCD and MS/2 media formed green secondary protonema, but to a lesser extent than the upright-oriented explants. Of all the media tested, the KNOP medium was suitable for the formation of massive green gametophores when the explants were positioned prostrate (Fig. 3B), and was therefore selected for further experiments. Plants grown on MS/2 medium did not develop new gametophores, although secondary protonema was documented, especially when the plants were positioned upright (Fig. 3C, F).

Table 1. Summary of factorial analysis for "Medium type experiment", evaluating the effects of explant position (EO), medium type (MT), and their interaction (EO × MT) on the index of multiplication (IM) and the diameter of the secondary protonema patch (DP) in *P. krylovii*. The values represent F-statistics, with asterisks indicating levels of statistical significance (**$P < 0.01$, ***$P < 0.001$).

Parameter	EO	MT	EO × MT
IM	7.3527**	51.0915***	1.7222
DP	24.6815***	5.2304**	12.2429***

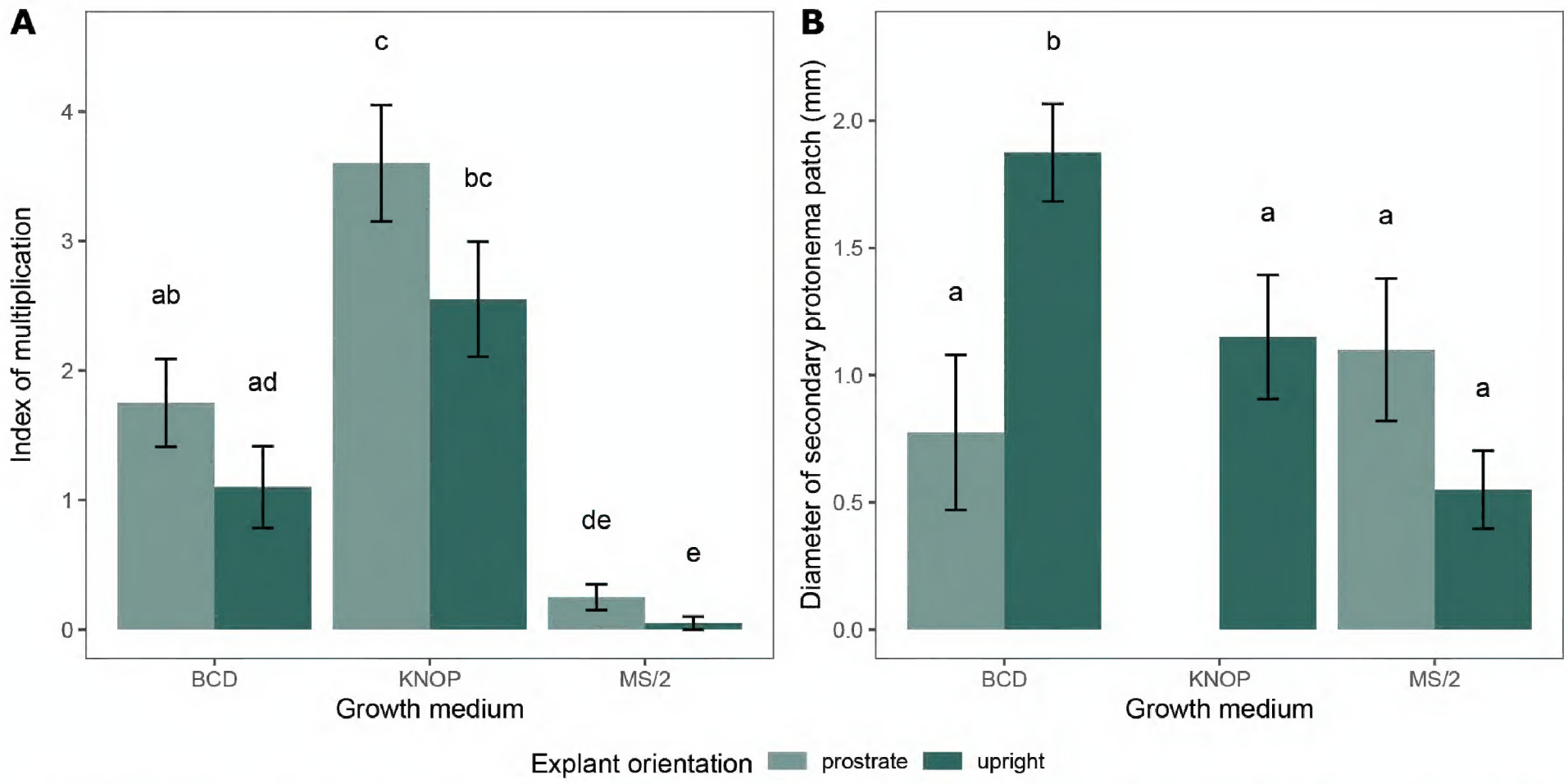

Figure 2. The effects of explant position and different growth media types on the index of multiplication (**A**) and the diameter of secondary protonema patch (**B**) were assessed in the "Medium type experiment". Data are presented as mean ± standard error. The letters above the bars represent statistically significant differences ($P < 0.05$) among the experimental groups.

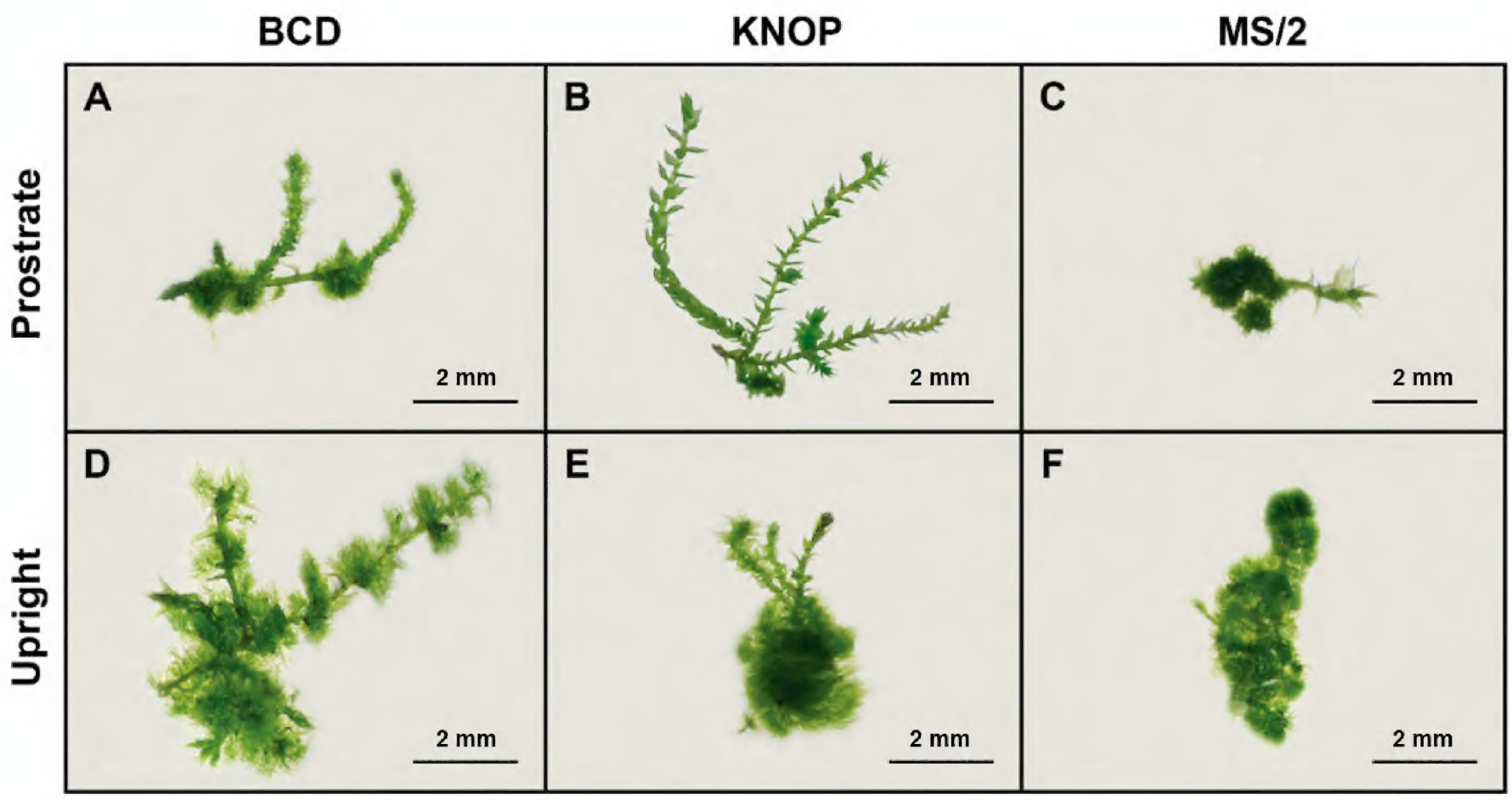

Figure 3. The appearance of *Podperaea krylovii* explants grown on different media types.

The effects of explant position and exogenous plant growth regulators on the morphogenesis of *Podperaea krylovii*

In the "PGR experiment", the effects of explant orientation (EO) and concentration (C) on morphogenesis parameters varied between IBA- and BAP-treated plants. In IBA-treated plants, explant orientation significantly affected both morphogenesis parameters evaluated ($P < 0.01$ and $P < 0.001$ for IM and DP, respectively), while

the effect of IBA concentration was significant only for DP ($P < 0.001$) (Table 2). Furthermore, a significant interaction between EO and concentration (EO × C) was observed for DP ($P < 0.001$), suggesting that the impact of IBA concentration on secondary protonema development is modified by explant orientation. However, no significance for such an interaction was found for IM.

On the other hand, in BAP-treated plants, the index of multiplication was significantly affected by BAP concentration ($P < 0.001$), but not by explant orientation (Table 2). Additionally, the diameter of the secondary protonema patch was significantly affected by EO, C and their interaction (EO × C) ($P < 0.001$) indicating a complex relationship between secondary protonema development, explant orientation, and BAP concentration (Table 2).

In the "PGR experiment", different plant growth regulators had different impacts on the morphogenesis of *P. krylovii* (Fig. 4). There were no statistically significant differences observed for the IM values in IBA-treated plants, although a slight, but not significant decrease can be observed in plants grown in an upright orientation compared to their prostrate counterparts for all of the applied IBA concentrations (Fig. 4A). On the other hand, IBA treatments influenced secondary protonema development differently depending on the explant orientation. In control plants with a prostrate orientation, there were no observed explants with developed secondary protonema (Fig. 4B). However, lower IBA concentrations (0.03 and 0.3 μM) strongly stimulated protonemal development, whereas the highest concentration (3 μM) also induced protonema formation, but resulted in significantly smaller DP values compared to the lower concentrations ($P < 0.05$) (Fig. 4B).

The index of multiplication of BAP-treated plants with a prostrate orientation decreased with increasing BAP concentration, with all the treated plants exhibiting significantly lower IM values compared to the control group ($P < 0.05$) (Fig. 4C). A similar trend of IM values decreases was observed in BAP-treated upright-oriented plants. However, in this case, only those plantlets treated with the highest applied BAP concentration (3 μM) exhibited significantly lower IM values compared to the control group ($P < 0.05$) (Fig. 4C). This suggests that the prostrate orientation could enhance BAP absorption due to the greater surface area contact with the medium. Secondary protonema patches were not observed in control plants with a prostrate orientation (Fig. 4D). However, the lowest applied BAP concentration (0.03 μM) successfully induced secondary protonema development in these plants (Fig. 4D). On the other hand, in an upright-oriented control plant, the highest DP values were observed and interestingly, treatment with any BAP concentration completely inhibited secondary protonema development in upright-oriented plants (Fig. 4D).

Table 2. Summary of factorial analysis for "PGR experiment", evaluating the effects of explant position (EO), concentration (C), and their interaction (EO × C) on the index of multiplication (IM) and the diameter of the secondary protonema patch (DP) in response to two plant growth regulators, IBA and BAP in *P. krylovii*. The values represent F-statistics, with asterisks indicating levels of statistical significance (**$P < 0.01$, ***$P < 0.001$).

PGR	Parameter	EO	C	EO × C
IBA	IM	7.15731**	0.88928	0.26528
	DP	16.1032***	9.8409***	16.8184***
BAP	IM	1.9112	19.5211***	2.6230
	DP	196.770***	33.474***	85.645***

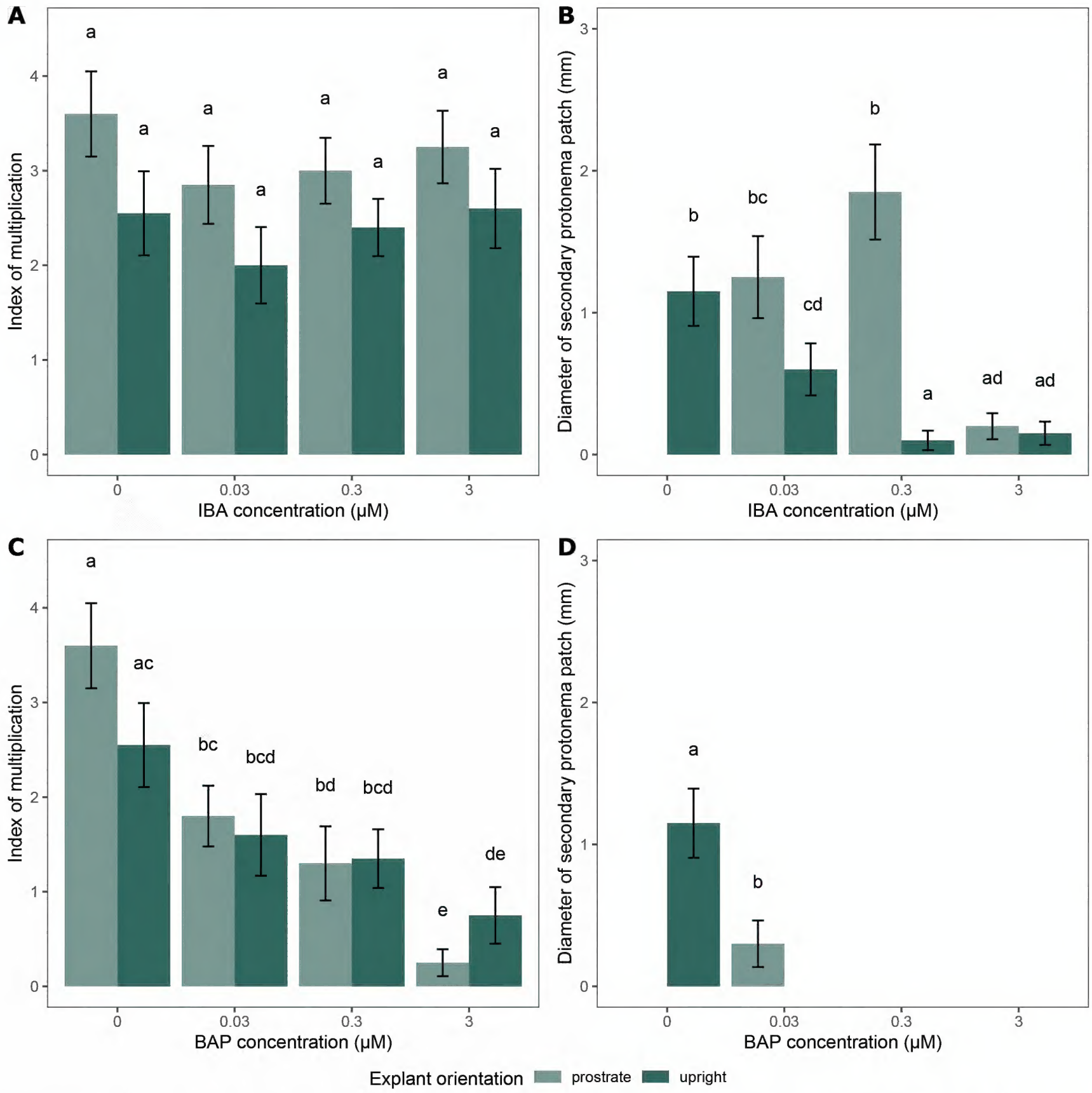

Figure 4. The effects of explant position and varying concentrations of IBA and BAP on the index of multiplication (**A** – IBA and **C** - BAP) and the diameter of the secondary protonema patch (**B** – IBA and **D** - BAP) were assessed in "PGR experiment". Results are presented as mean ± standard error. Statistically significant differences among experimental groups are indicated by different letters above the bars ($P < 0.05$).

The exogenously applied IBA had no effect on the formation of new gametophores when explants were positioned upright and prostrate (Fig. 5F). The newly developed gametophores are green, well-shaped, and similar in size to those of the control group (Fig. 3B). However, IBA promoted the formation of secondary protonema in the prostrate-positioned explants (Fig. 5A–C), particularly when applied as 0.3 μM (Fig. 5B), which was not observed in the control plants (Fig. 3B). At high concentrations, IBA inhibited further enlargement of secondary protonema diameter in *P. krylovii* (Fig. 5C). In contrast, when BAP was applied, plants developed normally in upright orientation, although failed to develop secondary protonema (Fig. 5J–L). The prostrate-oriented plants were smaller and thinner compared to the control plants and to IBA-treated plants (Fig. 5G–I). Interestingly, BAP inhibited the formation of secondary protonema in all plants except when applied at the lowest concentration (0.03 μM) (Fig. 5G).

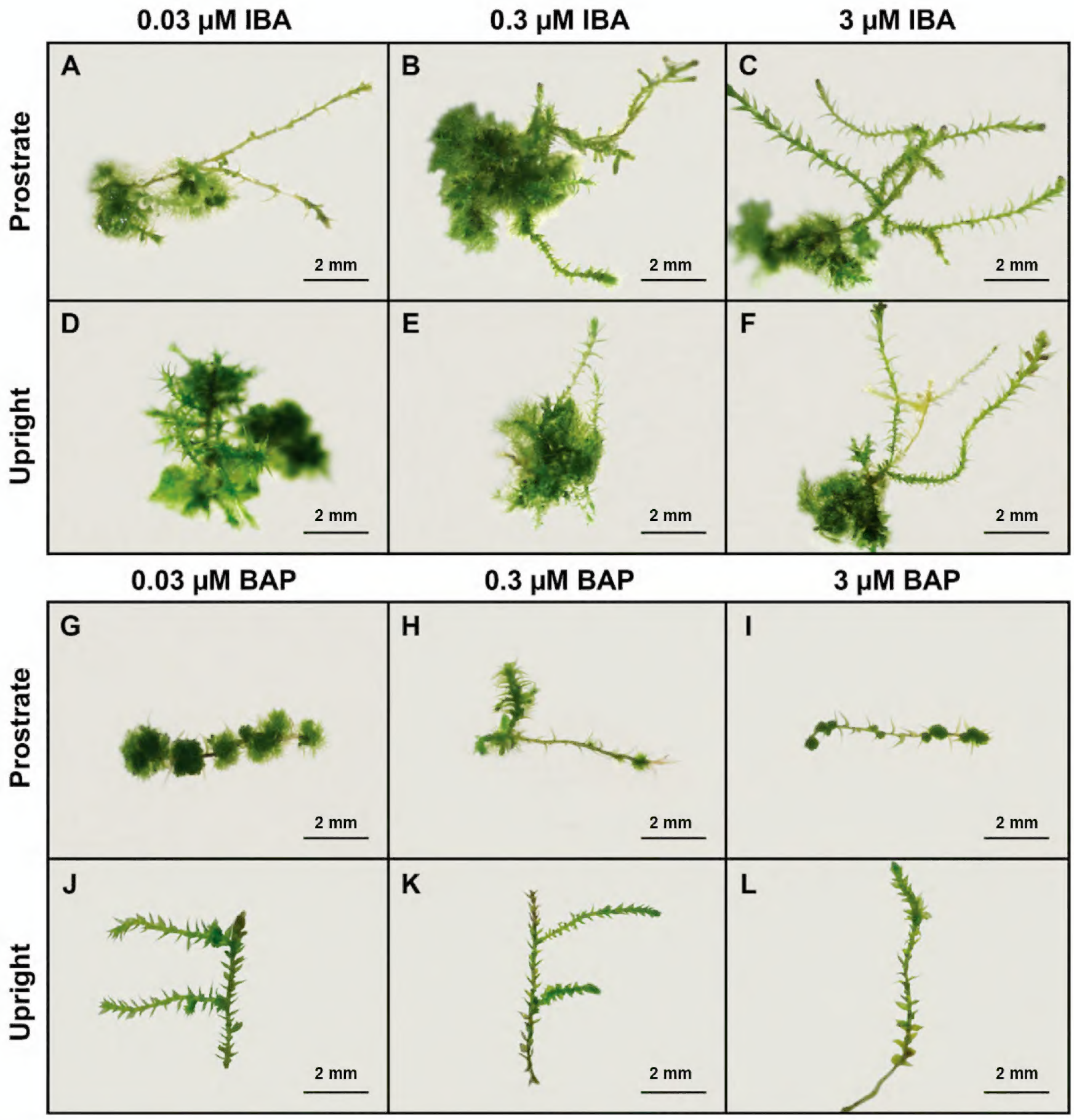

Figure 5. The appearance of *Podperaea krylovii* explants grown on KNOP medium supplemented with different IBA (**A–F**) and BAP (**G–L**) concentrations. Control group plants are shown in the Fig. 3B, E.

The effects of various sugar types on the morphogenesis of *Podperaea krylovii*

In "Sugar type experiment" both sugar type (ST) and sugar concentration (SC) had highly significant main effects ($P < 0.001$) on the index of multiplication of the species (Table 3). Additionally, the interaction between sugar type and concentration (ST × SC) was also significant ($P < 0.05$), suggesting that the effect of sugar concentration on the index of multiplication of *P. krylovii* varies depending on the type of applied sugar (Table 3). In the case of the diameter of secondary protonema, sugar type (ST) had a significant effect ($P < 0.001$), while sugar concentration (SC) did not show a significant main effect (Table 3). However, the interaction between sugar type and concentration (ST × SC) was significant ($P < 0.01$), implying that the effect of sugar concentration varies depending on the applied sugar type (Table 3).

Table 3. Summary of factorial analysis for "Sugar type experiment", evaluating the effects of various sugar types (ST), sugar concentration (SC), and their interaction (ST × SC) on the index of multiplication (IM) and the diameter of the secondary protonema patch (DP) of *P. krylovii*. The values represent F-statistics, with asterisks indicating levels of statistical significance (*P < 0.05, ***P < 0.001).

Parameter	ST	SC	ST × SC
IM	11.3580***	9.5074***	2.5797*
DP	10.17142***	0.37009	3.82904**

In the "Sugar type experiment", the exogenous addition of sugar (fructose, sucrose, glucose, and sucrose) to the KNOP medium significantly (P < 0.05) reduced the index of multiplication compared to the control group only in the case of lower applied concentrations (0.04 M) of glucose and sucrose and higher concentrations (0.08 M) of sucrose (Fig. 6A). Regarding the development of secondary protonema, different effects were observed depending on the sugar type applied. A significant increase in DP values compared to the control group was observed for both applied concentrations of sucrose (P < 0.05) (Fig. 6B). In contrast, a significant decrease in DP values was observed when a lower concentration of glucose and a higher concentration of fructose were applied (P < 0.05) (Fig. 6B).

The morphology of the plants treated with various sugar types was generally normal (Fig. 7A–H), although developed plants were remarkably smaller compared to the control plants (Fig. 3B). Overall, glucose severely inhibited the formation of new gametophores and secondary protonema when applied at a concentration of 0.04 M (Fig. 7B, F). In all other treatments, plants developed thick secondary protonema patches. Nevertheless, the formation of new gametophores was observed in plants grown on the KNOP medium supplemented with fructose and maltose, but those were remarkably smaller and less developed than the control group (Fig. 3E). However, these differences, although present, were not statistically significant (Fig. 6A). In contrast, glucose and sucrose had negative effects on IM, especially when applied at lower concentrations (0.04 M) (Fig. 7B, D).

Acclimation of *in vitro* propagated *Podperaea krylovii*

A translocation test was carried out from native sites and native substrates in the Partizansk district (Primorsky Territory, Russia) under controlled conditions, as well as the test of acclimation of *in vitro* produced plant material to xenic aquarium growth.

The plant material produced *in vitro* in the laboratory was tested *ex situ* in the laboratory (Fig. 8A, B) and also by transfer of xenic material from growth chambers outdoors (Fig. 8C). It was found that the plantlets grew very well when transferred to a new environment and left on the original substrate for some time. The plants were kept on loamy soil in plastic boxes that were covered to ensure high humidity and watered indiscriminately when necessary. The *ex situ* tests resulted in well-developed gametophytes, but the sporophytes produced appear to be morphologically rare and possibly of apogamic origin, as it has already been shown in some other mosses in which low light intensity can induce

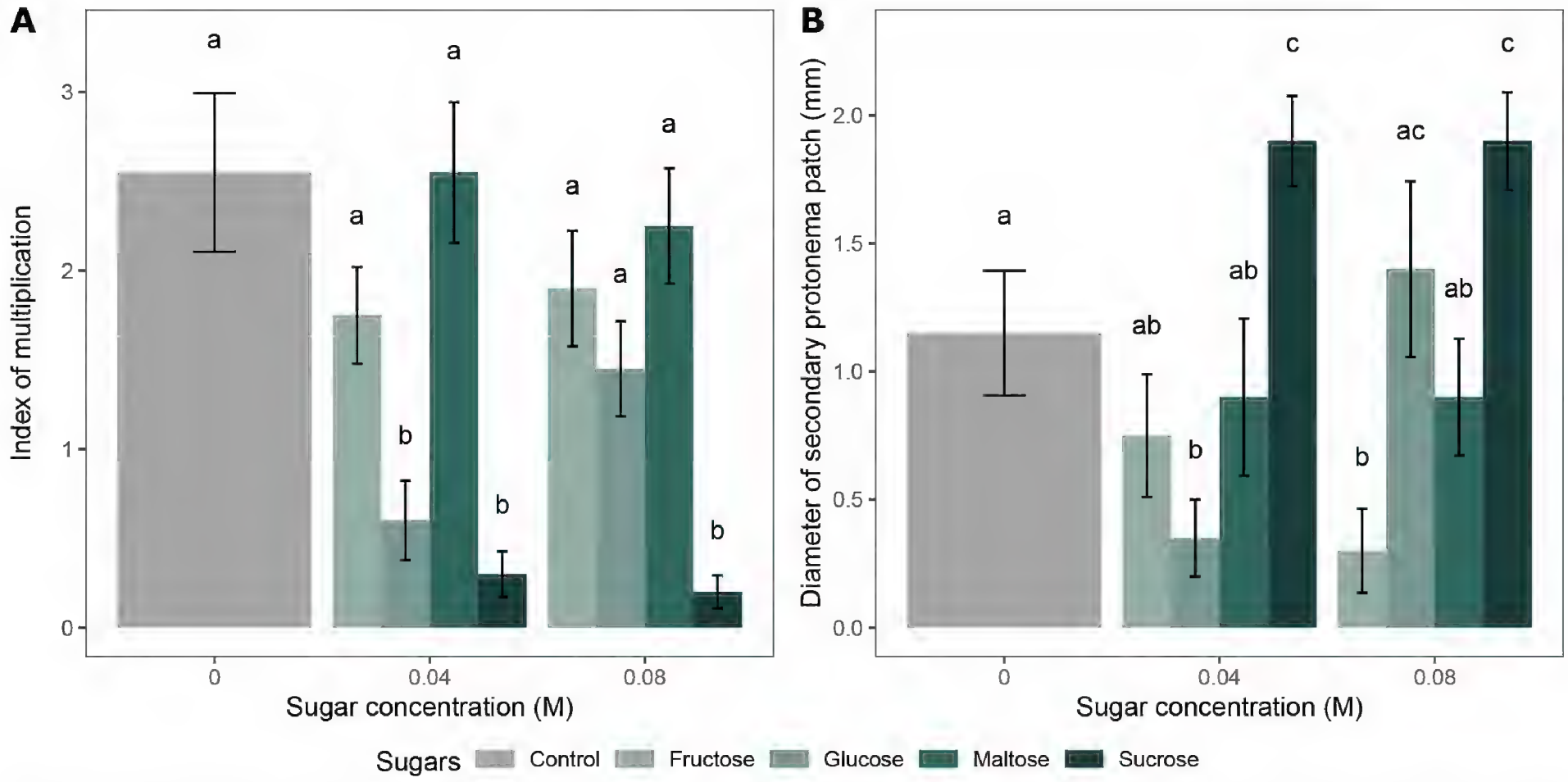

Figure 6. The effects of varying sugar concentrations and explant position on the index of multiplication (**A**) and the diameter of the secondary protonema patch (**B**) were assessed in the "Sugar type experiment". Results are presented as mean ± standard error, with statistically significant differences among experimental groups denoted by distinct letters above the bars ($P < 0.05$).

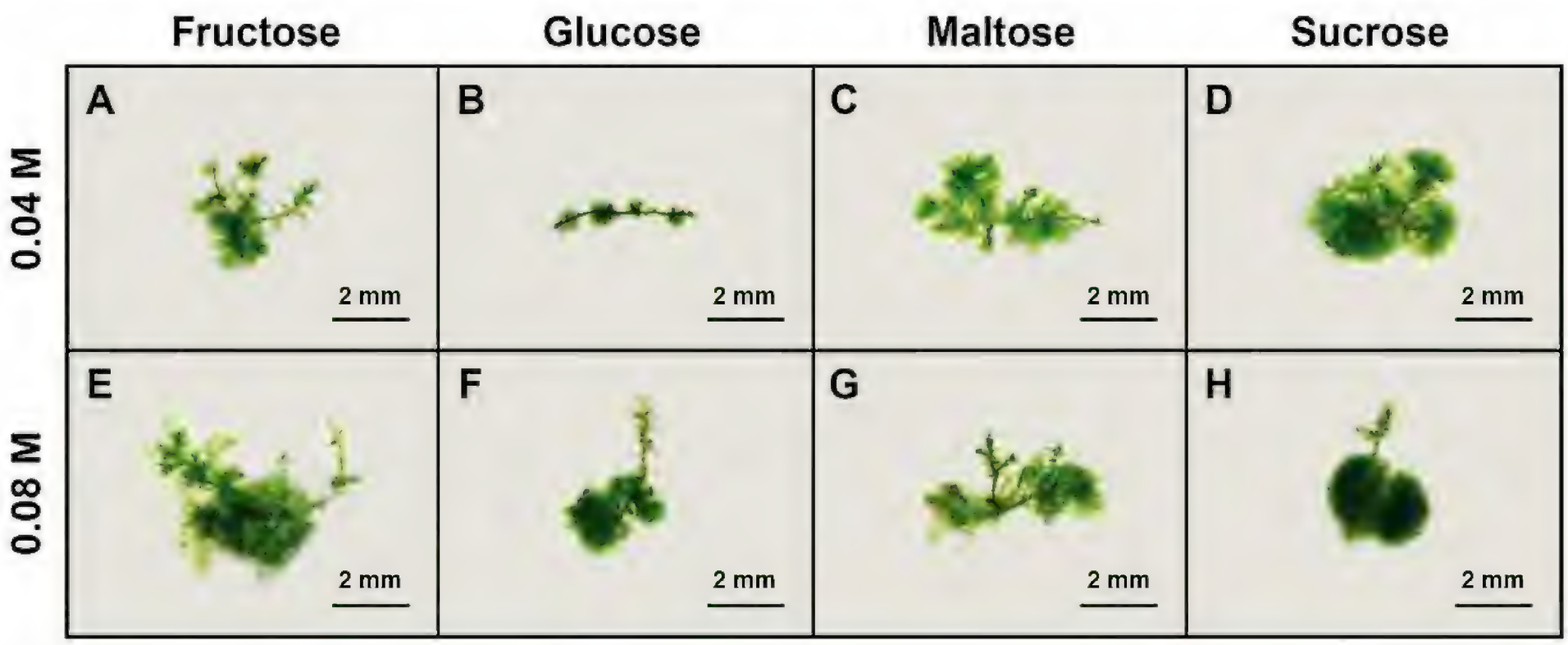

Figure 7. The appearance of *Podperaea krylovii* explants in upright position grown on KNOP medium supplemented with different sugar types and concentrations. Control group plants are shown in the Fig. 3E.

apogamy, both in Amblystegiaceae and other unrelated groups (Sabovljević et al. 2022 and the references therein). Numerous sporophytes of *P. krylovii* did not reach maturity, or produce spores and were also morphologically abnormal, suggesting apogamous development. Under these conditions, sporophytes often do not appear until 2 to 4 weeks after the start of the test.

Attempts were made to acclimatize the species to the water conditions for faster growth in aquaria filled with distilled water without aeration. The temperature was 18 ± 2 °C at a constant light condition of 35 µmol m^{-2} s^{-1} PPFD. The initial results are promising, as within four weeks the size of the plants in the water has increased many times over, and many new branches have emerged.

Figure 8. Aquarium acclimatization (indoor) of *P. krylovii* originated from *in vitro* (axenic) cultures (**A** – at the beginning, **B** – four weeks after); **C** *Ex situ* (xenic) propagation on native substrate (outdoor) from the material originated from nature with the morphologically strange shape of sporophytes, possibly of apogamous origin.

Discussion

Influence of growth medium type and explant position on the morphogenesis of *Podperaea krylovii*

Given the limited literature and data available on the selected species, studying the effects of different types of growth media on the morphogenesis of *P. krylovii* will lead to a better understanding of the growth and development of the species in axenic conditions. The temperature and selection of PGR concentrations were selected based on previous experience and to allow comparison with other species (Sabovljević et al. 2022 and the references therein). From the results obtained, it appears that of the three types of media tested, the solid KNOP medium was the most suitable for further propagation and multiplication; regardless of whether the plantlets were oriented prostrate or upright (Figs 2A, 3B, E). Prostrate-oriented explants grown on KNOP medium had a higher number of newly formed buds and did not develop any secondary protonema (Fig. 2B), which could indicate that this orientation is particularly favorable for *P. krylovii* gametophore development.

Additionally, prostrate-oriented explants are fully immersed in the culture medium on one side, allowing them to take up elements from the substrate more effectively. In contrast to *P. krylovii*, upright-oriented explants of another pleurocarpous moss species *Drepanocladus lycopodioides* (Brid.) Warnst (Jadranin et al. 2024) formed a higher number of new shoots, probably due to increased airflow around the explants, nutrient uptake through the wounded ends of the explants (Papafotiou and Martini 2009), and active cell-to-cell transport (Oliver and Bewley 1984). The results obtained in this study showed that higher biomass production (higher index of multiplication) is achieved when the surface of the plant is in close contact with the medium so that a larger surface area is involved in nutrient uptake. Compared to the prostrate-oriented explants, an upright orientation on the KNOP medium led to the formation of both secondary protonema and new shoots of *P. krylovii* (Figs 2A, B, 3E). In addition, plants developed new shoots also when grown on BCD medium, especially when positioned prostrate, albeit to a significantly lesser extent compared to plants grown on KNOP medium (Fig. 2A). However, the secondary protonema was smaller when explants were grown on BCD medium in a prostrate position compared to upright-oriented explants (Fig. 3A, D), which may indicate that the plant invests more resources in the development of gametophores when oriented prostrate.

On the contrary, MS/2 medium was not suitable for the propagation of *P. krylovii*, but similar to the other two tested media types, plants placed prostrate exhibited a slightly higher index of multiplication (Fig. 2A). In this case, the protonema developed in both upright- and prostrate-oriented explants (Figs 2B, 3C, F). Considering that *P. krylovii* inhabits wetlands with distinct microclimatic conditions, it is suggested that KNOP medium is well-suited for propagating *P. krylovii* due to its nutrient composition, which likely aligns with the specific nutritional requirements of the species. In addition, KNOP medium is widely used for the *in vitro* cultivation of a variety of bryophyte species (Ahmed et al. 2010; Liu et al. 2016; Sabovljević et al. 2022) such as mosses, liverworts (Awashti et al. 2013), and peat mosses (Såstad et al. 1998; Beike et al. 2014; Natcheva and Cronberg 2023). The BCD medium has proven to be less effective compared to KNOP, probably due to its specific composition and higher nitrogen content (KNO_3), which exceeds the needs of the selected species.

However, with minor modifications, this medium has been shown to be suitable for certain moss species such as *Hennediella heimii* (Hedw.) R.H. Zander, *Molendoa hornschuchiana* (Hook.) Lindb. ex Limpr., *Hypnum cupressiforme* Hedw. and *Entosthodon hungaricus* (Boros) Loeske, but it is not used as frequently as KNOP medium (Sabovljević et al. 2022 and the references therein). Interestingly, MS/2 was a suitable medium for the propagation of some species such as *Pterygoneurum sibiricum* Otnyukova (Jadranin et al. 2023), *Eurhynchium praelongum* (Hedw.) B.S.G. or also mentioned *H. cupressiforme* (Sabovljević et al. 2022 and the references therein). In this study, as in the case of *D. lycopoidoides* (Jadranin et al. 2024) or *Campyliadelphus elodes* (Lindb.) Kanda (Jadranin et al. 2025), MS/2 medium was not suitable for propagation. One of the reasons for this could be that the MS/2 medium probably does not meet the nutritional requirements of *P. krylovii* since it contains higher concentrations of nitrate ions derived from different mineral salts. Compared to the KNOP medium which contains only $Ca(NO_3)_2 \times 4H_2O$ as a nitrogen source, MS/2 is composed of two different mineral salts such as NH_4NO_3 and KNO_3. Therefore, it is suggested that additional ammonium ions can contribute to such adverse results. Although further detailed studies are necessary, these findings provide a solid basis for research on the selected species. A fundamental understanding of its nutrient requirements and habitat conditions could be crucial for future studies.

Influence of plant growth regulator and explant position on the morphogenesis of *Podperaea krylovii*

Previous studies (Ashton et al. 1979; Bhatla and Chopra 1981; Jadranin et al. 2023) have shown that plant growth regulators (PGRs) can influence development or increase the number of newly formed shoots in bryophytes. Cytokinins and auxins are likely to play important and interdependent roles in several steps of gametophytic development in bryophytes (Chopra and Sarla 1986; Anglana et al. 2024). To investigate the effects of IBA and BAP on the morphogenetic parameters of *P. krylovii*, different concentrations (0.03, 0.3, and 3 µM) were added individually to the basal KNOP medium on which the plant explants were placed both upright and prostrate. Although exogenously applied IBA led to the formation of new buds, none of the applied IBA concentrations resulted in a

higher number of newly formed buds (Fig. 4A) compared to the control treatment. These results indicate that KNOP medium without IBA addition is the most suitable for the propagation of *P. krylovii*, i.e. that this species forms a large number of new shoots regardless of the presence of PGRs. Similar findings were observed for *Physcomitrella patens* (Hedw.) Bruch & Schimp., which can spontaneously form a large number of new buds (von Schwartzenberg 2009). Exogenous application of auxins at times can disturb the endogenous level of these hormones and generally inhibit bud formation in mosses, particularly when used in higher concentrations (Sarla and Chopra 1987). It was previously found that in the case of decapitated moss *Plagiomnium cuspidatum* (Hedw.) T. Kop. exogenous auxins inhibited the formation of new buds on primary shoots (Nyman and Cutter 1981). However, in this study, none of the used IBA concentrations were inhibitory for the formation of new gametophores. For all IBA concentrations tested, prostrate explants had a higher index of multiplication than upright explants (Fig. 4A). This result also supports the assumption that the prostrate orientation enables efficient uptake of nutrients and hormones from the medium by *P. krylovii*. In contrast to the studies done on *D. lycopoidoides* (Jadranin et al. 2024), *Entosthodon pulchellus* (H. Philib.) Brugués (Ćosić et al. 2025b), and *P. sibiricum* (Jadranin et al. 2023), where IBA led to a significant decrease in the formation of new shoots, such a result was not observed in *P. krylovii*. Regarding the effects of IBA on the formation of secondary protonema, the results show that protonemal development occurs in prostrate-oriented explants, except at the highest IBA concentration where protonemal growth was partially inhibited. On the contrary, in upright-oriented explants, the diameter of the secondary protonemata decreased upon IBA treatment (Fig. 4B). Comparable results were obtained for certain mosses such as *P. sibiricum* (Jadranin et al. 2023), *E. pulchellus* (Ćosić et al. 2025b), and *Hennediella heimii* (Sabovljević et al. 2022 and the references therein) where PGR concentrations also led to a reduction in the diameter of secondary protonemata.

Influence of different types of sugars on the morphogenesis of *Podperaea krylovii*

Plant responses to sugars, both as energy sources and signaling molecules can differ among bryophyte species. Therefore, it would be beneficial to study different types of sugar to determine how the selected species will respond to their exogenous addition. Four types of sugar were tested at two different concentrations (0.04 and 0.08 M) to examine the response of *P. krylovii* to specific sugars. These sugars were incorporated into the basal KNOP medium, which had been found to be the most suitable medium in prior testing. According to the results obtained, only a lower concentration of glucose and both sucrose concentrations reduced the number of newly formed shoots (Fig. 6A, 7B, D, H). Glucose also reduced the diameter of secondary protonema while sucrose increased it. Similar results were obtained in a study on *D. lycopodioides* with the same types of sugars (Jadranin et al. 2024), suggesting that these species do not require additional sugars as an additional carbon source to increase their biomass. This can be explained by the fact that bryophytes in axenic culture, when exposed to light, actively grow with high photosynthetic activity (Katoh 1983; Božović et al. 2024), eliminating the need for an additional energy source.

Comparable results were observed in *Atrichum undulatum* (Hedw.) P. Beauv. where the applied sugars had an inhibitory effect on the development of new buds and also negatively affected the development of secondary protonema (Sabovljević et al. 2022 and the references therein). *Pterygoneurum sibiricum* exhibited equivalent results, with the highest number of new shoots forming when grown on basal KNOP medium, while the addition of sugar inhibited shoot formation (Jadranin et al. 2023). On the other hand, certain studies have reported that exogenously added sugars can positively impact the growth of particular species, highlighting species-specific differences in sugar utilization and physiological responses. Such results were observed in *Bryum argenteum* Hedw. (Sabovljević et al. 2022 and the references therein), where exogenously added sugars led to an increase in newly formed shoots. In addition, *Pogonatum urnigerum* (Hedw.) P. Beauv. and *Dicranum scoparium* Hedw. are species whose growth was positively influenced by the addition of sugars in the growth medium (Sabovljević et al. 2022 and the references therein). Experiments conducted on *Funaria hygrometrica* Hedw. and *Leptobryum pyriforme* (Hedw.) Wilson with sugars such as glucose, sucrose, maltose and fructose have shown that these species can grow in the dark when one of these carbon sources is available (Simola 1969), indicating the metabolic switch to heterotrophy. On the other hand, Katoh (1983) has shown that *Marchantia polymorpha* L. was not able to grow in the dark, even in the presence of a carbon source (glucose), due to reduced respiration levels. There have also been experiments in which sugar has induced sexual organs, such as in *B. argenteum* (Liang et al. 2010). However, in our study, no such phenomena were observed.

The gathered results suggest that the role of sugars in bryophyte development can vary significantly among species and that a deeper understanding of these species-specific responses will be crucial for optimizing growing conditions. Further research is needed to elucidate the potential role of sugars in the metabolism of selected moss species.

Increasing the BAP concentration consistently led to a decrease in the formation of new shoots in both upright and prostrate-oriented explants (Fig. 4C). Similar findings were observed in studies on *D. lycopodioides* (Jadranin et al. 2024) but also in the acrocarpous mosses *H. heimii*, *B. argenteum*, *A. undulatum*, and *P. cuspidatum* (Sarla and Chopra 1987; Sabovljević et al. 2022 and the references therein). In contrast to these results, a large number of newly formed gametophores were observed in *P. patens* when exogenous BAP was applied (Ashton et al. 1979). Secondary protonema was mostly inhibited when BAP was applied, except in the prostrate-oriented explants treated with the lowest concentration (0.03 μM) (Fig. 4D). These findings were previously documented for *Garckea phascoides* (Hook) C. Muller (Chopra and Sarla 1986), *E. pulchellus* (Ćosić et al. 2025b) and *P. sibiricum* (Jadranin et al. 2023). Such results suggest that BAP is not suitable for the propagation of *P. krylovii* under axenic conditions, as the plant can spontaneously produce more shoots without the addition of cytokinins. Besides its effects on the formation of protonemal and shoot buds in bryophytes, cytokinins seem to have a role in the formation of female sex organs (archegonia) at the tip of the gametophores, such was the case in the upright-positioned plantlets of *D. lycopoidoides* (Jadranin et al. 2024). An earlier study conducted on female clones of *Bryum argenteum* revealed that exogenous cytokinins increased the production of fertile gametophores, while

inhibited it in the male clone (Bhatla and Chopra 1981). Although BAP was not suitable for multiplication *in vitro*, its application was valuable for the induction of sex organs in the case of this pleurocarpous moss.

Overall, the development of new buds can be altered by exogenously added cytokinins and auxins, since there are complex interactions between endogenous hormones and the exogenous portion of PGRs. Therefore, due to the species-specific responses of bryophytes tested so far of the PGRs, further studies are required, making the determination of optimal concentrations a challenge. However, the results clearly indicate that *P. krylovii* can be propagated vegetatively and axenically on basal KNOP medium without the addition of PGRs. However, because of the various effects that PGRs can have in bryophytes, it is important to study their potential effects on the formation of some other structures besides the impact they have on morphogenetic parameters.

The results achieved in this study clearly introduce new knowledge to the biology as well as the *ex situ*/*in vitro* growth conditions of this rare species. However, the axenic cultures and propagation offer many further possibilities for investigations like biological activity and chemical constituents of this moss species as well as other biological features like biofiltration or toxic elements accumulation.

Conclusion

The rare and threatened moss *P. krylovii* was successfully propagated under axenic and xenic *ex situ* conditions and optimal conditions for biomass production were achieved. The study shows that the KNOP medium is most suitable for the propagation of *P. krylovii*, especially when the explants are placed in a prostrate orientation, which gives the highest index of multiplication. This orientation enhances nutrient uptake, resulting in better biomass production. The BCD medium supports growth, but its effectiveness is lower compared to KNOP. The MS/2 medium, on the other hand, is unsuitable as the plants invested more in the development of the secondary protonema and developed a lower number of new shoots. The addition of different concentrations of IBA had no effect on the index of multiplication, while the addition of BAP led to a lower formation of new shoots and inhibited the development of protonema. Furthermore, the tested sugars had no positive effect on the multiplication of an axenic culture of *P. krylovii*, and sucrose even reduced the number of newly formed shoots. Leafy gametophores were successfully used for acclimation and xenic *ex situ* propagation of the species.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Conceptualization, A.D.S. and M.S.S.; Methodology, M.V.Ć and M.M.V.; Software, D.P.B.; Validation, A.D.S., D.P.B., B.Z.J and M.S.S.; Formal analysis, B.Z.J., M.V.Ć and D.P.B.; Investigation, B.Z.J., M.V.Ć and D.P.B.; Resources, M.S.I, A.V.T and M.S.S.; Data curation, B.Z.J. and D.P.B.; Writing—original draft, B.Z.J., M.V.Ć and D.P.B.; Writing-Review and Editing, B.Z.J., M.V.Ć D.P.B. and M.S.S.; Visualization, B.Z.J., M.V.Ć and D.P.B.; Supervision, A.D.S. and M.S.S.; Project administration, A.D.S.; Funding Acquisition, M.S.S.

Author ORCIDs

Bojana Z. Jadranin https://orcid.org/0009-0001-4506-699X
Marija V. Ćosić https://orcid.org/0000-0002-3506-8705
Djordje P. Božović https://orcid.org/0000-0002-5816-5903
Milorad M. Vujičić https://orcid.org/0000-0002-2152-9005
Michael S. Ignatov https://orcid.org/0000-0001-6096-6315
Aleksey V. Troitsky https://orcid.org/0000-0002-0471-2633
Aneta D. Sabovljević https://orcid.org/0000-0003-3092-9972
Marko S. Sabovljević https://orcid.org/0000-0001-5809-0406

Data availability

All of the data that support the findings of this study are available in the main text.

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